

Carbon Dioxide & the Hill Reaction¹

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Recently a number of investigators have reported that the Hill reaction is more rapid in the presence of CO₂ than in its absence. However, the phenomenon of CO₂ dependence can only be demonstrated under certain conditions and it is therefore regrettable that there has been no agreement as to the nature of these conditions. Warburg and Krippahl (9) have stressed the importance of preilluminating the chloroplasts, whereas Abeles et al. (1) found the effect primarily in unbuffered media. Stern and Vennesland, on the other hand, observed large CO₂ effects in buffered media and without preillumination; in their studies the composition of the buffer was important (7). We have confirmed and extended the observations of the last-named authors. We have also investigated the relation of CO₂ dependence to light intensity, the state of uncoupling of the phosphorylation system, and the nature of the Hill reaction oxidant. The new information presented here provides little support for Warburg's contention that CO₂ plays a role in the Hill reaction akin to its role in photosynthesis. In fact it is inconsistent with some of the details of Warburg's postulated mechanism. However, the crucial question remains unanswered: Is CO₂ a metabolite in the Hill reaction or are we dealing with indirect and possibly unspecific effects of the bicarbonate ion?

Materials & Methods

Chloroplasts were isolated from young pea plants (*Pisum sativum* L. var. Alaska) by the following procedure: The leaves were ground in a chilled mortar with a buffer containing 135 g sucrose, 2.5 g bovine serum albumin, and 0.05 mole tris(hydroxymethyl)methylglycine-NaOH per liter, pH 7.5. The homogenate was filtered through glass wool into ice-encased centrifuge tubes. It was then centrifuged at 4000 × *g* for 3 minutes and the supernatant fluid discarded. The chloroplasts were resuspended in the same buffer, recentrifuged, taken up in a small volume of the buffer and stored in an ice bath. Small aliquots of the dense suspension were added to the reaction vessels.

Oxygen production was measured manometrically in standard Warburg vessels. Potassium ferricya-

nide, if used, was added from the side arm towards the end of the 2 to 3 hour incubation period. The preincubation took place in the dim light of a shuttered room. White light from six 300-w incandescent reflector lamps was used during the reaction. The intensity of the light, which was not measured, fell appreciably short of saturating the more active Hill reactions (see fig 5). The CO₂ concentration of the gas phase was regulated by the addition of CO₂ buffers to the center well. The CO₂ pressures in equilibrium with several of the buffers were measured with a mass spectrometer. These observed pressures agreed reasonably well with the pressures reported by Warburg and Krippahl (8) and with the pressures predicted by the law of mass action.

Because ferricyanide reduction in the Hill reaction leads to hydrogen ion formation, adequate CO₂ buffering and pH buffering are essential if the reaction is to be followed manometrically. In order to make the CO₂ buffer capacity as large as possible, the CO₂ buffers were very concentrated (usually about 2.0 M) and 0.4 ml instead of the usual 0.2 ml was used. Spilling over of this larger volume was prevented by the use of extra large paper fans in the center wells. Equilibration of the CO₂ buffer with the gas phase was catalyzed by the inclusion of 0.1 M sodium arsenite. [Arsenite catalyzes the hydration of CO₂ (6).] In some experiments CO₂ pressure was maintained by a 2.0 M KHCO₃, 2.0 M sodium arsenite solution mixed with 1.0 M diethanolamine. This gave 0.6% CO₂ in the gas phase and very rapid equilibration. When such precautions were taken, it was possible to assign practically all of the pressure changes in the vessels to O₂.

Since there were some anomalies in the reaction of the chloroplasts to the available 2,6-dichlorophenol-indophenol (DCPIP), purchased from the Eastman Organic Chemicals, it was suspected that the dye might contain impurities. Consequently it was purified in the following manner: by careful regulation of the pH, the dye was transferred repeatedly between chloroform (acid form) and water (salt form), then between ether and water. The last traces of ether were removed from the final water phase by aeration and the sodium salt of the dye was precipitated by the addition of controlled amounts of NaCl. This salting out procedure was repeated. The purified material and the original sample had the same optical density and the same effects on the Hill reaction.

Tris(hydroxymethyl)methylglycine (tricine) was

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prepared by reacting tris(hydroxymethyl)aminomethane (tris) with monochloroacetic acid as described elsewhere (3).

Results

Interactions of Bicarbonate with Other Anions.

The Hill reaction is influenced very much by the presence or absence of a number of different anions. Warburg first demonstrated that chloride could be essential to the photochemical activities of isolated chloroplasts (11). Subsequently, it was shown that chloride interacts with other anions in diverse ways, sometimes stimulating and sometimes inhibiting the Hill reaction, and that anions other than chloride react in a similar complex manner (3, 5). Consequently it seemed that the dependence of chloroplasts on CO_2 (which is mostly in the form of bicarbonate ion at the pH's used) might be very much modified by other anions. Perhaps some other anion could fulfill the function of bicarbonate in CO_2 depleted chloroplasts, thereby restoring the Hill reaction potential.

The results of a large and heterogeneous assortment of experiments, in which chloroplasts were pre-incubated for two to three hours with a variety of anions or mixtures of anions, may be summarized as follows:

The smaller monofunctional ions such as formate, acetate, chloride, and fluoride, have the effect of increasing the dependence of the chloroplasts on CO_2 . The same ions are only slightly inhibitory, if at all, when the CO_2 has not been removed. Formate and acetate are effective at much lower concentrations than chloride; maximum chloride enhancement of the effect of CO_2 removal is at concentrations which are osmotically unfavorable (0.3–0.5 M), while very significant acetate enhancement can be detected at concentrations below 10^{-2} M. Furthermore, there is synergism among these anions. For instance, acetate and chloride together depress the rate in the CO_2 depleted system more than would be predicted from their separate activities. Figure 1 illustrates some of the effects of chloride and acetate with acetate.

Glycolate and malate are the only two ions we have yet found to have any action in relieving the inhibition caused by CO_2 removal. Perhaps there is some significance in the fact that they resemble bicarbonate in having a hydroxyl group close to the carboxyl group. However, the stimulations caused by these two ions are hardly spectacular and do not nearly restore the full Hill reaction capacity observed in the presence of CO_2 . Thus in an acetate-chloride medium the rate is often more than 90% inhibited by the removal of CO_2 . Glycolate (10^{-2} M) increases this very low rate, but by not more than 40% instead of 1000%.

Glycolate also can be powerfully inhibitory when the Hill reaction oxidant is DCPIP, whether or not CO_2 is present. The anions of two other hydroxy acids with hydroxyls close to the carboxyl are always

inhibitory, whether or not either CO_2 or DCPIP is present; salicylate is strongly inhibitory at concentrations down to 10^{-3} M, while lactate is slightly inhibitory but only at much higher concentrations. Predictably, many other anions are also indiscriminately inhibitory, especially at high concentrations: iodide, arsenite, nitrate, fatty acids, etc.

A great many anions do not significantly affect the Hill reaction under the conditions of our study. This group consists mainly of large or polyfunctional ions such as citrate, oxalate, malonate, maleate, arsenate, phosphate, and pyrophosphate. It may be of some interest that the bulky trimethylacetate ion is inert although the slimmer propionate and butyrate ions resemble acetate in enhancing the effect of CO_2 removal. Substances with hydroxyls remote from the carboxyl group, such as p-hydroxybenzoate and D,L- β -hydroxybutyrate also have no effect on the Hill reaction. The same is true of the zwitterions glycine and tris(hydroxymethyl)methylglycine.

It should be emphasized that the above observa-

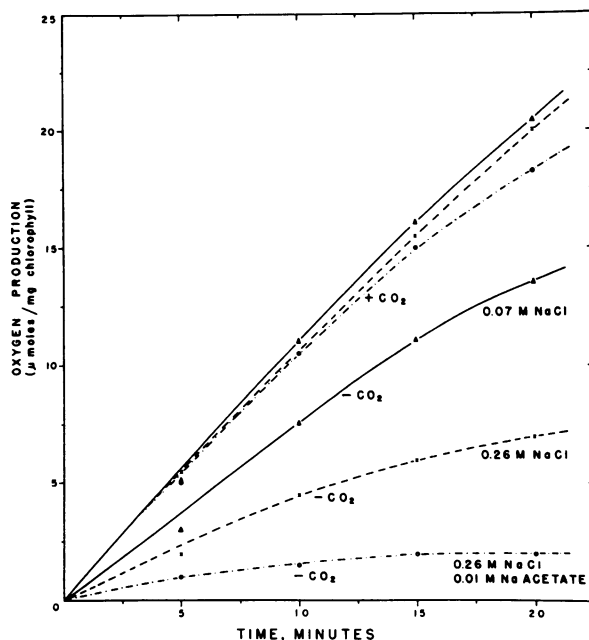


FIG. 1. The influence of chloride and acetate on the CO_2 dependence of the Hill reaction. Pea chloroplasts ($126 \mu\text{g}$ chlorophyll) were incubated for $2\frac{1}{2}$ hours at 16° in 0.05 M phosphate buffer containing the indicated concentrations of sodium chloride and sodium acetate plus 1.35×10^{-7} moles DCPIP. Final pH 6.6, final volume 1.9 ml. The center wells of the vessels contained either KOH or CO_2 buffer. The concentration of CO_2 in the gas phase in equilibrium with this CO_2 buffer (65 parts 2.0 M KHCO_3 : 35 parts 2.0 M K_2CO_3) was approximately 1.1%. Twenty micromoles of potassium ferricyanide in 0.1 ml water were tipped into the vessel from the side arm after the incubation period and before the period of illumination. The three lower curves illustrate the progress of ferricyanide reduction in the absence of CO_2 . The three upper curves are their controls with CO_2 .

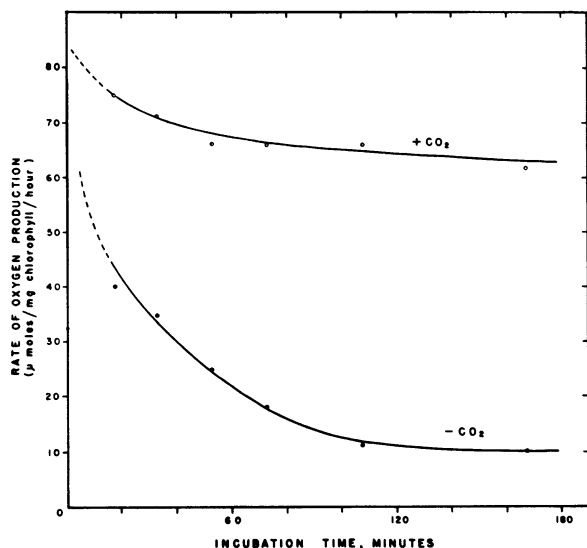


FIG. 2. The dependence of the chloroplasts on exogenous CO_2 as a function of the time of incubation. Pea chloroplasts ($130 \mu\text{g}$ chlorophyll) were incubated at 18° for the indicated times under air containing 0.0 or 0.6% CO_2 (KOH, or 50 parts 2.0 M KHCO_3 and 50 parts 2.0 M K_2CO_3 containing 0.1 M sodium arsenite, in the center wells). The reaction medium consisted of phosphate (0.05 M), sodium chloride (0.2 M), and sodium acetate (0.01 M) and contained 1.35×10^{-7} moles DCPIP. Final pH 6.55, final volume 1.9 ml. Twenty micromoles of potassium ferricyanide in 0.1 ml water were tipped into the vessel from the side arm immediately before the period of illumination. The time of incubation is taken as extending to the middle of the 15 minute period of illumination.

tions are applicable to pea chloroplasts incubated with shaking for at least two hours, usually under conditions designed to give the largest CO_2 depletion effects. Under more favorable conditions or with shorter times of exposure these anions might evoke quite different responses. Perhaps the use of other chloroplast sources and other preparative methods would lead to different results. However, there is good reason to believe that the spinach chloroplasts of Stern and Vennesland responded in the same way to chloride. These authors found that the dependence on CO_2 was greater with pyrophosphate buffer than with phosphate buffer (7). Since pyrophosphate buffer is ordinarily made by adding HCl to sodium pyrophosphate, their pyrophosphate buffer was almost certainly inadvertently high in sodium chloride.

Preincubation & Preillumination of Chloroplasts. Although the removal of CO_2 causes some inhibition in the shortest experimental periods consistent with conventional manometric techniques, a prolonged period of incubation under a CO_2 free atmosphere is necessary to bring about a maximum reduction in the Hill reaction rate (fig 2). The required time of about two hours is not shortened appreciably by continuous illumination at high light intensities and no greater CO_2 dependence develops as a result of such

illumination. In these respects we cannot duplicate the observations of Warburg and Krippahl (9).

Under our conditions of incubation carbonic anhydrase in the medium does not shorten the necessary incubation period. Consequently the requirement for a long preincubation probably reflects the slow dissociation of some CO_2 or bicarbonate complex, rather than the slow removal of the last traces of bicarbonate from the medium.

Reversibility of Inhibition Caused by CO_2 Removal. On first inspection the decline in Hill reaction capacity shown by the CO_2 depleted preparations in figure 2 might be interpreted as a gradual, irreversible inactivation of the chloroplasts under the influence of an unfavourable mixture of salts. Similarly the much smaller decline in the presence of CO_2 might be attributed to a protective effect of bicarbonate ion through some kind of ion antagonism. However, as Stern and Vennesland have reported (7) and we have confirmed, the effects of CO_2 removal are reversible if CO_2 is restored before the period of illumination. Therefore, the progressive decline illustrated in figure 2 cannot be due to a process which damages the chloroplasts over the full 2 hours. Nevertheless, it still might be argued that CO_2 removal sensitizes the chloroplasts to light injury, and Warburg's claim that preillumination is necessary to bring out the full effect of CO_2 removal would tend to support this argument. That such is not the case can be seen from the experiment shown in figure 3. In this experiment CO_2 depleted chloro-

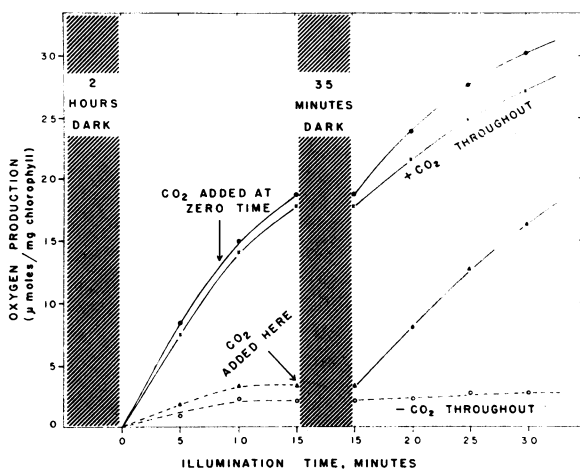


FIG. 3. Reversibility of the inhibitory effects of CO_2 removal. Pea chloroplast ($130 \mu\text{g}$ chlorophyll) were preincubated and illuminated in the reaction mixture described for figure 2. Some vessels (labeled + CO_2 throughout) were incubated and illuminated with a CO_2 buffer in the center wells (bicarbonate-arsenite-diethanolamine as described in text). Other vessels were incubated with KOH in small removable cups. These cups were left in the vessels ($-\text{CO}_2$ throughout), removed and replaced by CO_2 buffer after the incubation but before illumination (CO_2 added at zero time), or removed and replaced by CO_2 buffer after 15 minutes of illumination (CO_2 added here). Solid lines represent the presence of CO_2 , broken lines its absence.

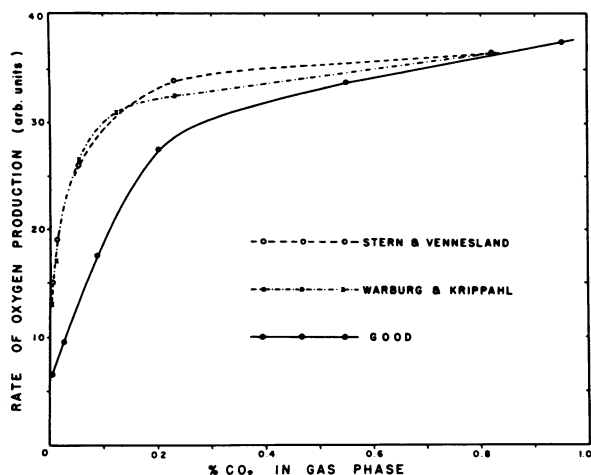


FIG. 4. The requirements of various Hill reaction systems for CO_2 . The data of Stern and Vennesland (7) and Warburg and Krippahl (9) are replotted with our data using arbitrary scales designed to make the curves coincide at high CO_2 pressures. In all of these experiments the CO_2 concentration was established by buffers containing potassium bicarbonate and potassium carbonate. The concentrations of CO_2 are either taken directly from the table provided by Warburg and Krippahl (8) or are computed from a constant derived from that table. Our reaction medium was the phosphate-chloride-acetate system described for figures 2 and 3, whereas the other data were obtained with media devoid of acetate and considerably lower in chloride.

plants lost all ability to produce O_2 after they had been illuminated in the presence of ferricyanide for a few minutes. Yet these chloroplasts recovered their full Hill reaction capacity when CO_2 was admitted to the gas phase.

Chloroplasts Activity as Function of CO_2 Concentration. The influence of various concentrations of CO_2 on the rate of ferricyanide reduction is shown in figure 4. In this figure the data of Warburg and Krippahl (9) for kohlrabi chloroplasts and the data of Stern and Vennesland (7) for spinach chloroplasts have been replotted along with our data for pea chloroplasts. The data of these two groups agree remarkably well, but we find a lower rate in the absence of CO_2 and a somewhat higher requirement for CO_2 . Both differences are probably related to the differences in the media: the chloroplasts used by us were exposed to acetate and high concentrations of chloride. Perhaps acetate and chloride compete with the bicarbonate ion for bicarbonate-dependent reaction sites on the chloroplasts.

Effects of pH & Buffer Composition on CO_2 Dependence. The chloroplasts used by Abeles et al. (1) exhibited a requirement for CO_2 only in unbuffered media where the pH was rather low (about 6.0). From their observation one might be tempted to attribute a critical local buffering role to CO_2 . Certainly around pH 6.0 the CO_2 -carbonic acid-bicarbonate shifts could provide the chloroplasts with

a very small amount of a highly mobile hydrogen ion buffer. However, the observations of Abeles et al. do not seem to be universally applicable. We usually find that the CO_2 requirement is independent of the composition, concentration and pH of the buffer if the active small anions described above (acetate, chloride, etc.) are avoided (see table I).

Table I

Effects of Various Buffers & pH on CO_2 Dependence of the Hill Reaction

Pea chloroplasts ($140 \mu\text{g}$ chlorophyll per vessel) were incubated in semi-darkness in the presence or absence of CO_2 (0.0 & 0.6 % in the gas phase) for 2 hrs at 18° . The incubation mixture contained the indicated buffer, 2×10^{-5} moles NaCl, and 1.35×10^{-7} moles DCPIP. Potassium ferricyanide (2×10^{-5} moles in 0.1 ml H_2O) was added from the side arm after the incubation period. Final volume 2.0 ml. Phosphate buffer was prepared by mixing KH_2PO_4 and Na_2HPO_4 solutions. Tricine buffer was prepared by adding NaOH to tricine. Tris buffer was prepared by adding H_2SO_4 to tris. The inhibitory nature of tris buffers & the superiority of tricine buffers is emphasized by the long incubation period.

pH	Buffer	Buffer concentration	Rate of O_2 production ($\mu\text{moles/mg chl/hr}$)	
			$-\text{CO}_2$	$+\text{CO}_2$
6.8	phosphate	0.05 M	92	108
		0.2 M	74	90
	tricine	0.2 M	74	89
	tris	0.2 M	3	6
7.5	phosphate	0.2 M	39	49
		0.05 M	74	89
	tricine	0.2 M	70	81
	tris	0.05 M	3	3
		0.2 M	3	4

Nature of Oxidant and Degree of Uncoupling. Flavin reduction is much less sensitive to CO_2 removal than is the reduction of ferricyanide. (See table II.) This difference could be the expression of a fundamental difference in the nature of the electron transfer in the two types of Hill reaction, or it could be simply the result of a profound difference in the reaction conditions. We varied the conditions of preincubation, using air or nitrogen in the gas phase and small amounts of ferricyanide or ascorbate in the incubation medium. Then the effect of CO_2 removal on ferricyanide and FMN reduction was determined for each condition of preincubation. The only outcome of these experiments was the observation that ascorbate under a nitrogen atmosphere improves the stability of the chloroplasts somewhat. However, as has already been pointed out, the effect of CO_2 removal is fully reversible and therefore one should not expect to find critical differences associated with the incubation history of the chloroplasts.

Table II

CO₂ Dependence of Hill Reaction as Function of Oxidant & Degree of Uncoupling

Pea chloroplasts (144 μg chlorophyll/vessel) were incubated for 2 hrs at 18° in semi-darkness, in a medium consisting of phosphate (0.05 M), NaCl (0.20 M), sodium acetate (0.01 M), pH 6.55. The center well contained either KOH or the potassium bicarbonate-sodium arsenite-diethanolamine buffer described in the text. Ferricyanide (2.0×10^{-5} moles in 0.1 ml H₂O) was added from the side arm after the incubation period. FMN (2.5×10^{-6} moles) and DCPIP (1.35×10^{-7} moles) were added before the incubation period. Final volume 2.0 ml.

Reaction conditions	Rate of O ₂ production ($\mu\text{moles/mg chlorophyll/hr}$)			
	-CO ₂		+CO ₂	
Ferricyanide as oxidant. Chloroplasts' electron transport system spontaneously & only partially uncoupled from phosphorylation	12,	16	33,	44
Ferricyanide as oxidant. Electron transport fully uncoupled from phosphorylation with 0.045 M methylamine	12,	13	61,	66
Ferricyanide & DCPIP as oxidants. Reduction of DCPIP is not coupled to phosphorylation	10,	12	61,	69
FMN as oxidant. Electron transport uncoupled from phosphorylation with 0.045 M methylamine*	-36,	-35	-62,	-65

* Since the reduced flavin reacts with molecular O₂ to form hydrogen peroxide, the Hill reaction results in a net uptake of O₂ equal to the amount of O₂ actually produced. Catalase and 2.0 % alcohol were added as a hydrogen peroxide trap in the flavin system (4). The amounts of catalase and alcohol used had insignificant effects on the ferricyanide containing systems.

The pertinent experiments would be to test the actual reduction of ferricyanide in a reducing medium and reduction of flavins in an oxidizing medium. Such experiments are not technically feasible.

When the Hill reaction is severely limited by the absence of CO₂, the rate is not influenced by agents such as amines and DCPIP which uncouple electron transport from the phosphorylation of ADP. The simplest interpretation of this phenomenon presupposes a linear sequence of reactions with phosphorylation and the CO₂ sensitive reaction occurring at different stages. Thus we must postulate that the phosphorylation step is rate-limiting when CO₂ is present since we know that uncouplers of phosphorylation stimulate the overall rate. We suggest that the overall rate in the absence of CO₂ is unaffected by the presence of uncouplers since the phosphorylation step no longer presents a bottleneck; leakage by it is more than sufficient to handle the trickle of electrons getting past the step inhibited by CO₂ removal. If this interpretation is correct, it follows that the process inhibited by CO₂ removal is not a reaction which is directly or obligatorily coupled to phosphorylation.

CO₂ Dependence & Light Intensity. As we have seen, CO₂ influences the Hill reaction rate when the light intensity is saturating or nearly saturating. However, CO₂ also influences the amount of light required to achieve saturation (see fig 5). When the photochemical process determines the rate, the Hill reaction rate is much less CO₂ dependent, and may well be completely independent. If this is so, the process inhibited by CO₂ removal is neither the primary photochemical reaction nor any reaction which indirectly affects the maximum photochemical efficiency of the Hill reaction.

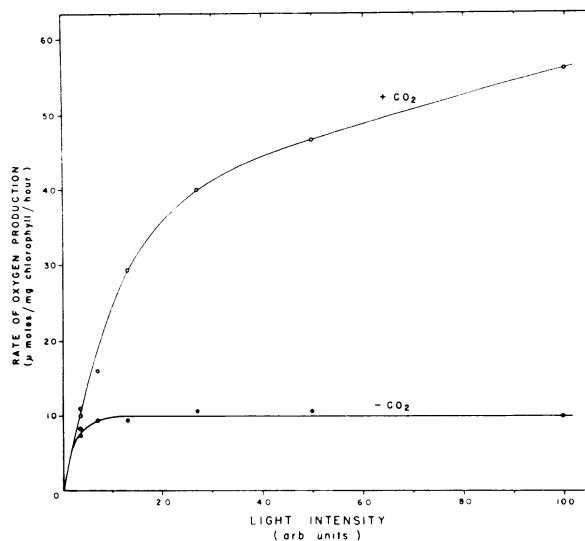


FIG. 5. Light intensity and the CO₂ dependence of the Hill reaction. Reaction conditions were as in figures 2, 3, and 4. The lower light intensities were obtained by wrapping the vessels with increasing numbers of layers of permanganate dyed cheese-cloth. The light transmission of each multilayer was determined with a light-meter while the cloth was immersed in water. The fact that the transmission of light diminished in a precisely logarithmic fashion with increasing numbers of layers was taken as proof of the accuracy of the measurements of relative intensities.

Discussion

In the conventional picture of photosynthesis, CO₂ fixation and reduction are thermochemical processes

which utilize TPNH and ATP to elaborate a variety of reduced metabolites, via reactions which are at least analogous to the reversal of respiratory dehydrogenations and decarboxylations. This concept is supported by a wealth of evidence: Labeling experiments employing $C^{14}O_2$ have shown that most of the intermediates of photosynthetic CO_2 fixation are also well known respiratory intermediates; the interconversions of these intermediates demonstrably required TPNH and ATP; finally, illuminated chloroplasts reduce pyridine nucleotides while simultaneously phosphorylating ADP to ATP. It is implicit in the conventional theory that the photochemical reduction of TPN or other oxidants by chloroplasts precedes and is fundamentally independent of CO_2 metabolism.

Warburg does not agree that the oxido-reduction reactions of chloroplasts are, in fact, independent of CO_2 metabolism. Indeed it is almost impossible to reconcile the above widely accepted interpretation of the Hill reaction with Warburg's scheme of photosynthesis, which postulates a photochemical process yielding one molecule of O_2 and consuming one molecule of CO_2 per quantum followed by a thermochemical back-reaction consuming the greater part of the released O_2 and releasing the greater part of the consumed CO_2 . (2). Therefore Warburg proposes the following radically different interpretation of the Hill reaction: the oxidant, CO_2 , water, phosphoric acid, and illuminated chlorophyll collaborate in some manner to form phosphorylpercarbonate. The phosphorylated peroxide of carbonic acid is hydrolysed and decomposed into oxygen, formic acid, and phosphoric acid and a second molecule of oxidant then oxidizes the formic acid to CO_2 . Thus it is held that CO_2 functions as a metabolite and yet catalytically (9,10).

As evidence in support of this complex and explicit new hypothesis, Warburg presents two related observations: A. the Hill reaction is inhibited by the exhaustive removal of CO_2 , and B. the concentration of CO_2 required for the Hill reaction is the same as that required for photosynthesis. While such evidence scarcely seems adequate as the sole experimental basis for an elaborate and detailed hypothesis, it is certainly legitimate to ask broader questions: Is CO_2 actually an intermediate in the Hill reaction? If so, does it play the same role in the Hill reaction as in photosynthesis? Or does CO_2 have, fortuitously, two unrelated functions in photosynthesis, a catalytic or stabilizing function as a cofactor in the oxido-reduction reactions of the chloroplasts and a non-catalytic function as the ultimate electron acceptor and the major substrate for autotrophic metabolism? Before attempting to answer these questions, we must consider Warburg's two pieces of evidence carefully.

The indispensibility of CO_2 cannot be established unless it can be shown that no other substance can be substituted for CO_2 and that the conditions required to produce CO_2 dependency are the conditions necessary for CO_2 removal or neutralization. No

systematic attempt to demonstrate either of these points has been reported to date. But even if we were to grant the indispensibility of CO_2 , it by no means follows that CO_2 must be a metabolite. Specific ions are frequently necessary for the maintenance of structural integrity in biological systems and for the activities of many enzymes. One might reasonably expect that bicarbonate ions would have some unique interactions with proteins; they have a negative charge for ionic bonding to the numerous positive sites on proteins juxtaposed to a hydroxyl group available for hydrogen bonding to the even more numerous carbonyl oxygens. In this connection it is interesting to recall that several other anions with hydroxyls close to the carboxyl have significant effects on the Hill reaction.

Warburg and Krippahl (9) have pointed out that the Hill reaction and photosynthesis require similar concentrations of CO_2 , thereby implying a common role for CO_2 . Indeed, their data for photosynthesis by kohlrabi leaves and for ferricyanide reduction by kohlrabi chloroplasts are strikingly similar. However, there is some doubt as to the significance of the comparison. When photosynthesis proceeds at an appreciable rate, the concentration of CO_2 at the site of its utilization must be lower than at the surface of the leaf. With decreasing external CO_2 concentrations this difference between the observed external CO_2 concentrations and the pertinent internal concentration will become a larger and larger proportion of the total. Although Warburg has attempted to minimize the complication introduced by diffusion lags, using very low light intensities with a consequent low rate of CO_2 uptake, there is still no assurance that the concentration of CO_2 inside the leaves bore any predictable relation to the known concentration outside. Consequently, the fact that the processes in the chloroplasts and in the leaves seem to saturate at about the same level of external CO_2 may not be meaningful. Moreover, there is another complication in interpreting this comparison of the Hill reaction and photosynthesis. It is true that the same levels of CO_2 will be required to saturate a common site whether it be in chloroplasts or in leaves, but saturation of photosynthesis by CO_2 rarely indicates saturation of the CO_2 binding enzyme. Ordinarily, photosynthesis rates fail to increase with rising CO_2 concentrations because some reaction subsequent to CO_2 binding becomes saturated. This is shown by the fact that the CO_2 saturation level of photosynthesis is usually a function of light intensity. Indeed, it is very probable that the similarity of CO_2 requirements noted by Warburg and Krippahl applies only to the light intensities they chose.

Considering the primitive nature of all our ideas concerning the processes going on in illuminated chloroplasts, how should we marshal the arguments for and against Warburg's conception of the role of CO_2 in the Hill reaction? No single argument is conclusive and, assembled, the arguments are not

very convincing on either side. Probably the best reason for countenancing interpretations of the kind put forward by Warburg is a proper abhorrence of relying on explanations which invoke coincidental relationships. Certainly believing that CO_2 has two unrelated functions in photosynthesis—or one function in photosynthesis and another trivial function in isolated chloroplasts—presents us with a truly disconcerting coincidence. In addition, some of the more obvious explanations which would assign a trivial function to CO_2 in the Hill reaction fail to accommodate the fact that the effect of CO_2 removal is completely reversible. However, there are also good reasons for not yet accepting Warburg's picture of the Hill reaction. The fact that CO_2 dependence seems to be correlated with the presence of small anions implies that bicarbonate ion is the important substance, not CO_2 . If this is so, the phenomenon resembles ion antagonism, not photosynthetic CO_2 metabolism. Schemes such as Warburg's, which postulate the reduction of CO_2 followed by its re-oxidation by the Hill reaction oxidant, predict equal or greater CO_2 dependence when the oxidant is a weak oxidant such as FMN; actually FMN reduction is very much less dependent on the presence of CO_2 than is ferricyanide reduction. The specific scheme put forward by Warburg et al. (10) suggests that a photochemical reaction is the process which is inhibited by CO_2 removal; on this point the authors are explicit "Dabei müssen alle Zwischenprodukte mit der Chlorophyll verbunden sein . . ." Yet we have seen that the photochemical efficiency of the Hill reaction is probably not impaired by CO_2 removal. Finally, Warburg would have us believe that photophosphorylation is intimately associated with CO_2 metabolism. Our data suggest that the effect of CO_2 removal involves a reaction remote from phosphorylation.

Summary

I. Pea chloroplasts incubated for two to three hours under a CO_2 free atmosphere may lose the greater part of their Hill reaction capacity.

II. This loss of Hill reaction capacity in bicarbonate-depleted chloroplast suspension is correlated with the presence of other small anions. Fairly high concentrations of formate, acetate, fluoride, or chloride must be present before removal of CO_2 results in a major reduction in the activity of the chloroplasts.

III. Phosphate, pyrophosphate, arsenate, citrate, maleate, malonate, trimethylacetate, p-hydroxybenzoate, glycine, and tris(hydroxymethyl)methylglycine (tricine) do not affect the Hill reaction rate, either in the presence or absence of CO_2 .

IV. Anions of alpha- and orthohydroxy acids such as glycolate and salicylate have a variety of effects, usually inhibiting the Hill reaction whether

or not CO_2 is present but sometimes partly relieving the inhibitory effects of CO_2 removal.

V. The effect of CO_2 removal is completely reversible. Chloroplasts which have been incubated with chloride and acetate in the absence of CO_2 , then illuminated with ferricyanide, rapidly lose all ability to produce oxygen. When such inactive chloroplasts are exposed to CO_2 , they fully recover their Hill reaction capacity.

VI. Ferricyanide reduction is much more dependent on the presence of CO_2 than is the reduction of FMN.

VII. There is evidence which suggests that the reaction inhibited by CO_2 removal is neither a photochemical reaction nor a reaction which is directly coupled to phosphorylation.

Acknowledgments

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